Integrated Master in Bioengineering

Spray dried propolis to improve the microbiological safety of alheira

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Resumo

O objetivo deste estudo foi a obtenção de um extrato seco de própolis e a sua incorporação em alheira com o objetivo de melhorar a sua segurança microbiológica.

O extrato etanólico de própolis foi seco por atomização (*spray dryer*), usando goma Arábica e maltodextrina como agentes de encapsulação em dois rácios diferentes, 1:4 e 1:6 (m/m). Foram determinados o rendimento do processo e a perda de compostos fenólicos totais do própolis, durante a secagem. A atividade antibacteriana do extrato seco de própolis foi qualitativamente demonstrada contra as bactérias *Listeria innocua*, *Staphylococcus aureus*, *Escherichia coli* e *Salmonella* Typhimurium. A eficácia do extrato seco de própolis foi avaliada no controlo de *L. innocua* e *Staph. aureus* durante o armazenamento da alheira a 4 °C durante 60 dias. Foram testadas diferentes proporções do pó adicionado à alheira: 1, 2, 5 e 10 % (m/m). Nas proporções de 1 e 2 % não houve inativação significativa ($P > 0.01$) das bactérias em estudo, em comparação com o controlo. O extrato seco de própolis adicionado à alheira num rácio de 5 % revelou ser eficiente no controlo de *L. innocua* e *Staph. aureus*, uma vez que o comportamento destas bactérias foi significativamente afetado ($P < 0.01$) pela sua presença. Foi feita uma análise sensorial de forma a avaliar o impacto da adição de extrato seco de própolis à alheira, na proporção de 5 %, tendo-se concluído que o sabor amargo característico do própolis não foi completamente mascarado pelo agente encapsulante.

A produção de própolis na forma de pó apresenta-se como uma alternativa viável ao extrato etanólico de própolis para aplicações na área alimentar e a sua adição na alheira demonstrou melhorar a sua segurança microbiológica. Contudo, de forma a permitir a adição de pó de própolis em alheiras, apresentando efeito antibacteriano sem alterar as propriedades organoléticas deste alimento, devem ser feitas melhorias no futuro.

Palavras chave: alheira, própolis, segurança microbiológica, *spray drying*.

Abstract

The aim of this study was to obtain a spray dried propolis extract (SDPE) and its incorporation in *alheira* in order to improve its microbiological safety.

Propolis ethanolic extract was spray dried using gum Arabic and maltodextrin as carriers in two different weight ratios (1:4 and 1:6). The yield of the process and the total phenolic loss of propolis during atomization were evaluated. The antibacterial activity of the spray dried propolis was qualitatively demonstrated against *Listeria innocua*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* Typhimurium. The efficacy of the SDPE was evaluated in the control of *L. innocua* and *Staph. aureus* during storage of *alheira* at 4 °C for 60 days. Different proportions of the powder in *alheira* were tested: 1, 2, 5 and 10 % (w/w). In the proportions of 1 and 2 % there were non-significant differences ($P > 0.01$) in the behaviour of the bacteria, comparing with the control. The SDPE added to *alheira* at a ratio of 5 % revealed to be efficient in the control of *L. innocua* and *Staph. aureus*, since the behaviour of these bacteria were significantly affect ($P < 0.01$) by its presence. A sensorial analysis was done to evaluate the impact of the SDPE in *alheira* in a ratio of 5 % and the characteristic bitter flavour of propolis was not masked by the carrier agent.

Propolis in a powder form is a suitable alternative for the ethanolic extract of propolis for food applications and its addition to *alheira* revealed to improve its microbiological safety. However, improvements should be done in order to allow the addition of propolis powder in *alheira*, exhibiting antibacterial effect, without changing the organoleptic properties of this food.

Key words: *alheira*, microbiological safety, propolis, spray drying.

Declaração

Eu, Catarina Sá Couto de Oliveira Fernandes, declaro, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

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List of abbreviations

ANOVA	Analysis of variance
CFU	Colony-forming unit
EEP	Ethanolic extract of propolis
GA	Gum Arabic
GAE	Gallic acid equivalent
GRAS	Generally Recognized as Safe
MD	Maltodextrin
MSA	Mannitol Salt Agar
OSA	Octenyl succinic anhydride
SDPE	Spray dried propolis extract
TPC	Total phenolic compounds
TSB	Tryptone Soya Broth
TSB-YE	Tryptone Soya Broth with Yeast Extract
TSA-YE	Trypto-casein Soy Agar with Yeast Extract
USA	United States of America

General Introduction

Background and project presentation

Fermented meat products are, in general, a considerable part of the daily diet in Portugal. Among other, *alheiras* are traditional Portuguese sausages that stand out due to their unique characteristics, as the composition and typical taste.

The thermal processes involved in *alheiras* production along with its chemical properties as the low pH, low moisture content and high salt content generally allow to consider them as microbiological safe foods. However, studies about chemical and microbiological safety characterization of samples of *alheiras* from different producers in Portugal reported by Ferreira *et al.* (2006, 2007) showed that, in most cases *alheira*s were produced under deficient hygienic conditions leading to post-process contamination after the thermal processes. According to the guidelines for the microbiological quality of fermented meats, published by Gilbert *et al.* (2000), most of the samples tested for the referred authors would be considered unsatisfactory, mainly due to the presence of high levels of *Enterobacteriaceae*, *E. coli* and *Staph. aureus*. *Listeria monocytogenes* was also found in a high percentage of the samples, which is a concern since this bacterium is responsible for a severe illness in humans (Skandamis & Gounadaki, 2007).

To overcome the problem of microbiological safety of *alheiras* and giving the increasingly demand for natural food additives, instead of the synthetic ones, propolis arises as a proper option. Propolis is a chemically complex resin collected by bees and due to its antioxidant and antimicrobial properties, it is considered a good natural preservative.

A previous study of the research group of Prof. Paula Teixeira dealing with the incorporation of ethanolic extract of propolis (EEP) in *alheira* was done (data not published at the present date) in which the efficacy of EEP in the control of *L. innocua*, as a surrogate for *L. monocytogenes*, *Clostridium sporogenes* and *Clostridium difficile* was evaluated, during storage of *alheira* at 4 °C. The behaviour of all bacteria in the food matrix was significantly affected ($P < 0.01$) by the addition of EEP. These results were the highlights for the studies developed in this work.

However, application of EEP in the food industry is still limited because it has a strong and unpleasant taste and aroma. Microencapsulation has emerged as a great solution to mask the taste of propolis and at the same time to obtain an alcohol-free propolis in a solid form, desirable in some situations (Silva *et al.*, 2011).

Thus, the main goal of this work was to obtain a spray dried propolis extract (SDPE) and its incorporation in *alheira* in order to improve its microbiological safety. The antimicrobial activity of SDPE was evaluated against *L. innocua* and *Staph. aureus* during storage of *alheira* at 4 °C for 60 days. Different proportions of the SDPE in *alheira* were tested: 1, 2, 5 and 10 % (w/w). Additionally, the yield production and the phenolic loss of the EEP formulations during the spray drying process were determined. Finally, a sensorial analysis was done in order to evaluate the impact of the SDPE incorporated in *alheira*.

Work organization

The developed work was divided into three different but complementary levels. The first one comprised the microencapsulation of EPP by spray drying in which different formulations of EEP and the carrier agent were tested. The production yield of the spray drying process, the phenolic loss of the SDPE and the antibacterial activity of the microcapsules produced were evaluated.

In the second part, the SDPE produced, which gathered the most favourable characteristics meanwhile assessed, was incorporated in the food matrix (*alheira*) in different proportions. The efficacy of the SDPE against *L. innocua* and *Staph. aureus* was evaluated. The final purpose was to optimize the proportion of propolis powder in *alheira.*

Finally, *alheira* samples with SDPE incorporated in the proportion that exhibited the most favourable results (highest antibacterial activity), were submitted to a sensorial analysis. For the possible application of SDPE in the food industry, which is the main motivation for the developed work, the organoleptic properties are crucial.

Organization of the Dissertation

This thesis is divided into five chapters. The first one comprises a Bibliographic Review related with the fundamental aspects of propolis characterization and microencapsulation; the spray drying technique; and the characterization of *alheiras*, mainly regarding their microbiological safety. Chapter two refers to the Materials and Methods and includes the reagents and equipment used as well as the procedures followed in the developed work. The chapter three is entitled Results and Discussion and presents all the relevant data obtained, its analysis and discussion taking into account the available reported work in this field. Chapter four involves the general Conclusions about the work and, lastly, chapter five is dedicated to the Perspectives of future work.

1. Bibliographic Review

1.1. Propolis' characterization

1.1.1. Composition and properties

Propolis or bee glue consists in a chemically complex resin collected by bees from the species *Apis mellifera* from different parts of plants such as sprouts, flowers, buttons, and resinous exudates (Ghisalberti, 1979, cited in Nori *et al.*, 2011). It is used by bees in the construction and maintenance of their hives (Burdock, 1998). Propolis' typical appearance is shown on Fig. 1.

Fig 1. Physical appearance of raw propolis (from Bio organic, available in: www.bio-organic.fr, accessed on: 12/04/2015).

In general, propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris (Burdock, 1998). At least 200 compounds were identified in different propolis samples, among them approximately 80-100 vary in different types of propolis according to its botanical and phytogeographical origin (Marcucci *et al.* 2001; Tosi *et al.*, 2006). Due to this fact, the "standardization" of propolis is very difficult. Among its components are included: fatty and phenolic acids and their correspondent esters, substituted phenolic esters, flavonoids (flavones, flavanones, flavonols, dihydroflavonols, chalcones), terpenes and sesquiterpenes, beta-steroids, aromatic aldehydes and alcohols, naphthalene and stilbene derivatives (Aga *et al.*, 1994; Bankova *et al.*, 1995; Marcucci *et al.*, 1996).

Simple fractionation of propolis to obtain the desired compounds is difficult due to its complex composition. The usual manner is to extract the fraction soluble in

alcohol, usually by ethanolic extraction, and the balsam thus obtained contains the bulk of propolis bioactive constituents (Marcucci *et al.*, 1995; Kalogeropoulos *et al.*, 2009).

Due to its composition, mainly the richness in phenolic compounds, propolis exhibits important biological and therapeutic actions (Lahouel *et al.*, 2004), reason why it has traditionally been used as a folk medicine with antiseptic and antibacterial properties since ancient times (Marcucci *et al.* 2001; Kim *et al.*, 2008). Besides the mentioned properties, propolis presents many other beneficial biological activities such as antifungal, astringent, choleric, spasmolytic, anti-inflammatory, anesthetic, antioxidant, healing, antiviral and anticarcinogenic (Marcucci, 1995; Burdock, 1998; Banskota *et al.*, 2001, cited in Silva *et al.*, 2013). Bearing in mind this so wide spectrum of beneficial properties, it is not surprising that it has been used as a natural additive and in the form of a functional ingredient in several pharmaceutical and food formulations (Nori *et al.*, 2011).

1.1.2. Propolis in the food industry

In recent times, the growing interest of the food industry to find natural additives has increased the efforts both in obtaining bioactive compounds from natural raw materials and in developing stable and functional derivative products (Silva *et al.*, 2013). This trend is fuelled by the increasing consumer awareness for natural, minimally processed foods with synthetic preservatives absent or at low concentrations (Tosi *et al.*, 2007). The properties attributed to propolis, combined with the fact that several of its constituents are present in food and/or food additives recognized as Generally Recognized as Safe (GRAS) (Burdock, 1998), makes it an attractive candidate as a natural preservative in the food industry, contributing with a positive effect on food product stability, shelf-life and safety (Silva *et al.*, 2013).

However, although the several products containing propolis which have been sold worldwide, such as candies, chocolates, sweets, shampoos, skin creams, antiseptic solutions, toothpastes and others, the application of propolis in the food industry is still limited mainly because it is has a strong and unpleasant taste and aroma (Nori *et al.*, 2011).

1.2. Propolis microencapsulation

In recent years some solutions have been proposed to overcome the previously mentioned limitations of propolis and allow its use in foods, besides the usual presence of ethanol in the propolis extract, which can be undesirable in some applications.

Amongst the various techniques suggested, including incorporation of EEP into edible coatings described by Pastor *et al.* (2011) and Torlak *et al.* (2013), microencapsulation has emerged as the more hopeful solution. Microencapsulation is a unique process used to convert liquids to solids to add functionalities and/or improve oxidative stability of the compounds. Masking the unpleasant flavours and odours of microencapsulated ingredients is one of its advantages, a reason why the application of this technique to propolis was considered. It becomes extremely important for the food industry to be possible the addition of this component to food without changing its characteristics, namely, organoleptic properties such as flavour, colour and texture. The main goal is help to improve the acceptability of beneficial although unpleasant-tasting ingredients (Winston, 2013). This is consistent with the increasing demand to find suitable solutions that provide benefits to the foods, high productivity and at the same time satisfy the required quality of the final products (El-Abassi *et al.*, 2014).

In this process, the active substance is surrounded by a membrane or embedded in a homogeneous or heterogeneous matrix (Ghosh, 2006). The material inside the microcapsule is referred to as the core, internal phase, or fill, whereas the wall is sometimes called a shell, coating, or membrane (Umer *et al.*, 2011). In the last two decades, applications of microencapsulation have increased in an exponential form (Suave *et al.*, 2006; Ghosh, 2006; Champagne & Fustier, 2007; Estevinho *et al.*, 2013a).

Propolis microencapsulation has been described by some authors. Bruschi *et al.* (2003) described a production process of propolis microparticles through spray drying using gelatin as a carrier agent. The spray dried propolis maintained the original antimicrobial activity suggesting that spray drying can be a promising process for developing an intermediary or eventual propolis dosage form without ethanol or a strong, unpleasant taste. However, in recent years the use of gelatin with an animal origin has been banned in some countries, thus alternatives should be considered. Nori *et al.* (2011) described the microencapsulation of propolis extract by complex coacervation using isolated soy protein and pectin as encapsulating agents. The study

concluded that it was possible to encapsulate propolis extract by complex coacervation and to obtain it in the form of powder, alcohol-free, stable, with antioxidant and antimicrobial activity and with the possibility of controlled release in foods. A method of propolis microencapsulation by spray drying using gum Arabic and octenyl succinic anhydride (OSA) starch-based carrier systems was described by Silva *et al.* (2013). In this study, propolis in a powder, alcohol-free and water-dispersible form was produced and its physical and functional properties were evaluated. The active agent with gum Arabic and OSA starch showed to be stable during storage at room temperature. More recently, Yang *et al.* (2014) described a method of encapsulation of propolis flavonoids in a water soluble polyethylene glycol using pressurized carbon dioxide anti-solvent co-precipitation process.

1.3. Microencapsulation in the food industry

In this section, an overview about the benefits of the microencapsulated formulations in the food industry is presented, regarding to the advantages and possible disadvantages of this technique.

Like other techniques of controlled release, microencapsulation allows the reformulation of a large number of products, mainly in the food and pharmaceutical industry, improving and giving them better and new properties as bioactive roles in the body (Pothakamury & Barbosa-Cánovas, 1995; Patel & Patel, 2010).

The industrial production of food often requires the addition of functional ingredients. Normally, these are used to control the physicochemical properties of the food like flavour, colour, texture or preservation properties, but ingredients with potentially healthy properties are also being increasingly included. However, adding bioactive ingredients to functional food presents many challenges, particularly with respect to the stability of the bioactive compounds during processing and storage and the need to prevent undesirable interactions with the carrier food matrix. Microencapsulation of bioactive ingredients is used to solve some of these problems. Another concern is that for obtaining a health benefit some actions are required to ensure the stability of the compounds, particularly in the human gastrointestinal system, and to facilitate controlled release. Again, the microencapsulation technique may be useful for this purpose (Champagne & Fustier, 2007).

All the benefits of microencapsulation in the food industry should overcome some possibly negative aspects, namely, additional costs, increased complexity of the production process and supply chain, undesirable consumer awareness of the encapsulates in the food, and stability challenges of encapsulates during processing and storage of the food product (Zuidam & Shimoni, 2010).

Costs deserve special mention in the food industry, since costs considerations are much more stringent than in the pharmaceutical or cosmetics industries, for instance (Desai & Park, 2005; cited in Estevinho *et al.*, 2013a). The applicability of a microencapsulation process in the food industry must be weighed against the final price of the product and its benefits. If it is an expensive ingredient, the cost-in-use should be lower than the non-encapsulated ingredient, but, if microencapsulation improves the properties of the ingredient, then the cost-in-use can be slightly higher than the non-encapsulated ingredient (Gouin, 2004).

Nevertheless, since encapsulates facilitate formulation of foods that are healthier, tastier and more convenient, the demand for this technique has been growing during the last decades (Frost & Sullivan, 2005). During the last years, the number of food compounds microencapsulated has been increasing, namely: flavours (Madene *et al.*, 2006; Estevinho *et al.*, 2013b), dyes (Ersus & Yurdag, 2007), stabilizers (Guillard *et al.*, 2009), antioxidants (Gemili *et al.*, 2010), enzymes (Sangeetha *et al.*, 2008; Estevinho *et al.*, 2012), probiotics (Champagne & Fustier, 2007; Heidebach *et al.*, 2010), lipids (Kralovec *et al.*, 2012), mineral salts (Oneda & Ré, 2003) and vitamins (Romo-Hualde *et al.*, 2012), among others.

1.4. Spray drying technique

Despite the high number of techniques developed to microencapsulate food ingredients, spray drying is the most common technology used in the food industry due to its low cost, versatility and equipment availability (Ghasallaoui *et al.*, 2007; Lamprecht & Bodmeier, 2012). It has been used in the food industry for several decades, being one of the oldest encapsulation methods. The main limitations of this microencapsulation technology are the limited number of wall materials available, which should possess good solubility in water, besides to be certified for food applications as GRAS (Ghasallaoui *et al.*, 2007). Also, the possibility of heat

degradation of the compounds due to the temperatures achieved during spray drying is another limitation to consider (Mujumdar, 2011).

In the next sections, a brief overview of the spray drying technical considerations and microencapsulation process steps is made. For a more in-depth review it is advisable to consult an excellent review paper published not long ago by Ghasallaoui *et al.* (2007), which provided the main support for the next sections.

1.4.1. Spray drying technical considerations

Spray drying is a unit operation by which a liquid product in the form of a solution, emulsion or suspension, is atomized in a hot gas current to instantaneously obtain a dry powder. In Fig. 2 a schematic diagram of the spray drying process is showed, which is thoroughly explained in the next paragraphs.

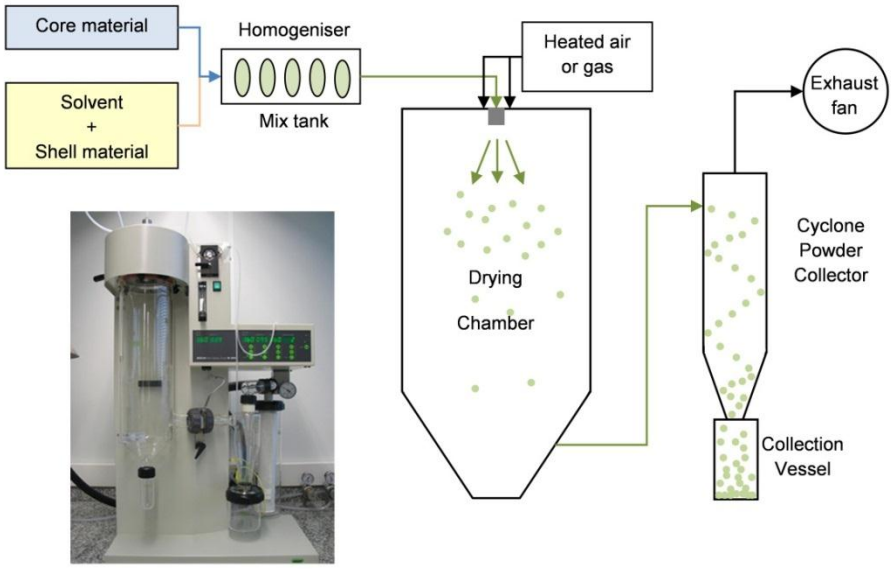

Fig 2. Schematic diagram of a spray drying encapsulation process and image of a Mini Spray Dryer B-290 (BÜCHI) (from Chávarri *et al.*, 2012).

The gas commonly used is air but an inert gas as nitrogen can also be used. The size of the particles formed can be very different, like a fine powder (10-50 μm) or large size particles (2-3 mm), depending on the starting feed material and operating conditions (Ghasallaoui *et al.*, 2007). In this process, active materials with differing solubility properties can be encapsulated with various wall materials.

When compared to other multi-step encapsulating processes, spray drying is a continuous single step operation, which has been scaled up for industrial applications

(Lamprecht & Bodmeier, 2012). Water removal by spray drying is used in food industry to ensure microbiological stability of products, avoiding chemical or biological degradations, reducing the storage and transport costs and obtaining products with some specific characteristics (Ghasallaoui *et al.*, 2007).

The spray drying process can be divided into four stages which happen almost simultaneously, namely, atomization, droplet-hot air contact, evaporation of droplet water and dry product-humid air separation.

The first stage involved in the spray drying technique is the atomization. Liquid atomization in small droplets can be carried out by pressure or centrifugal energy. Depending of the nature and viscosity of the feed as well as the desired properties of the dry particles, pneumatic atomizers, pressure nozzle, spinning disc configurations, two fluid nozzle or sonic nozzle can be used (Masters, 1968; cited in Ghasallaoui *et al.*, 2007).

The purpose of the atomization process is to provide a maximum heat-transferring surface between the dry air and the liquid, optimizing heat and mass transfer. The size of the particles formed depends on several factors: the higher the energy provided, the finer are the particles; formed particles increase with increasing feed rates; the higher the viscosity and surface tension of the liquid are, the larger are the particles (Ghasallaoui *et al.*, 2007).

The second stage is droplet-hot air contact and takes place during the preceding stage and it is the beginning of the drying stage. It can happen in two different ways: co-current or counter-current process, if the liquid is sprayed in the same direction of the flow air or in opposite directions, respectively. In the first one, the typical temperatures of the hot inlet air are in a range of 150-220 ^{0}C but the final powder will be exposed to lower temperatures (50-80 ^{0}C), which limits thermal degradation. In the second one, the dry product is exposed to higher temperatures which limit the application of this process to thermo-sensitive products. However, counter-current drying is significantly more economic in term of consumed energy (Ghasallaoui *et al.*, 2007).

The evaporation of droplet water occurs due to balances of temperature and vapor partial pressure between liquid and gas phases, responsible for the heat transfer from the air through the droplet and water transfer in the opposite direction.

Just after the contact between the hot air and the liquid, the droplets temperature increases to a constant value, defined as wet bulb thermometer temperature; after that the loss of moisture is controlled by the gas phase resistance. In this period, called constant-rate period, the rate of water diffusion from the droplet core to its surface remains nearly constant, as well the temperature and the partial pressures of water vapor. As drying continues, a gradient in water concentration builds up within the drop, the water activity at the surface decreases and thus the surface dries out (Ré, 1998).

When the droplet water content reaches a critical value, a dry crust is formed and the drying rate decreases. Since this moment, the drying process depends on the water diffusion rate through this crust. The drying is theoretically finished when the droplet temperature equals the hot air temperature. The duration of these three steps depends on both product nature and air inlet temperature. Due to the large surface to volume ratio of the droplets, the drying process is very rapid. Usually, the process takes approximately 5 to 100 seconds, (Corrigan, 1995) although in a well-designed system, 15 to 30 seconds can be achieved. Thus, spray drying is considered a rapid method for drying compounds which degrade when dried by a slower method (Ghasallaoui *et al.*, 2007).

The last stage of the spray drying consists in the separation of the dry product from the humid air. It is normally achieved with a cyclone placed outside the dryer. The densest particles are recovered at the base of the drying chamber, while the finest ones pass through the cyclone. In addition to cyclones, spray dryers are often equipped with filters, used to remove the finest powders, and chemical scrubbers, to remove the remaining powder or any volatile pollutants (Ghasallaoui *et al.*, 2007).

To decrease the drying temperature, increasing the residence time, multi-stage spray dryers are available. The advantage is to avoid the thermal denaturation of products, while the thermal effectiveness is increased (Schuck, 2002).

1.4.2. Microencapsulating process steps

In the previous section, the general process of spray drying was described regarding to its technical aspects. In this section, considerations about the entire process of microencapsulating by spray drying are presented, as well the operating conditions required.

The first step in this process involves the preparation of an emulsion containing the core and wall materials. Normally this is done through a dispersion of the core material, usually hydrophobic, in the coating agent, wherewith is immiscible.

Then, the emulsion is heated and homogenized; an emulsifier can be added in this step if necessary. Before the spray drying process, the emulsion must be stable over a certain period of time (Liu *et al.*, 2001). Emulsion viscosity should be low and oil droplets small, since these parameters have a great influence on the microencapsulation process.

After that, the emulsion is atomized and the solvent evaporates, which allows forming the microcapsules with a spherical shape wherein the oil phase is encased in the core material (Dziezak, 1988).

1.4.3. Operating conditions

In order to obtain good microencapsulation efficiency as well as the desired properties of the capsules, the operating conditions of spray drying should be optimized. The main factors that can be tuned are feed temperature and inlet and outlet air temperatures.

Regarding the feed temperature, it has an important role controlling the emulsion's viscosity and fluidity and thus, its capacity to be homogeneously sprayed. The temperature should be sufficiently high but without causing volatilization of the liquid, or degradation of the heat-sensitive compounds.

The temperature of the inlet air is proportional to the microcapsule drying rate as well as the final content of water. Thus, the temperature cannot be too low, at the risk of forming microcapsules with a high density membrane, high water content, poor fluidity and a tendency for agglomeration. On the other hand, high temperatures result in cracks in the membrane inducing subsequent premature release and a degradation of encapsulated ingredients or also a loss of volatiles (Zacarian & King, 1982).

The temperature at the end of the drying zone can be considered as the control index of the dryer. It is difficult to predict this temperature in advance for a given product, since it depends on the drying characteristics of the material. However, the ideal outlet air temperature reported for the microencapsulation of food ingredients is between 50 and 80 ^{0}C (Ghasallaoui *et al.*, 2007).

1.4.4. Wall material selection

The choice of a wall material is of great importance for encapsulation efficiency and stability. The wall system is designed to protect the core material against adverse conditions like light, pH, and oxygen activity in order to prevent possible deterioration, premature interaction with other ingredients, volatile losses and also to allow controlled or sustained release under desired conditions (Shahidi & Han, 1993; cited in Ghasallaoui *et al.*, 2007).

The encapsulating material is selected according to the properties of the active agent (porosity and solubility) and of the encapsulating agent, the compatibility between both (the wall material should be insoluble and not react with the core), the application required, the intended size for the microcapsules as well economic factors (Freiberg & Zhu, 2004; Azeredo, 2005; Suave *et al.*, 2006; Ghosh, 2006; Gharsallaoui *et al.*, 2007). Another important consideration is that the encapsulating material should have a low hygroscopicity to allow easier handling and not present an unpleasant taste, this last factor being of extreme importance in the food industry (Cortesi, Nastruzzi & Davis, 1998; Favaro-Trindade *et al.*, 2010).

Physico-chemical properties of the compounds are the main criteria for the selection of the encapsulating agent in spray drying, namely, solubility, molecular weight, glass/melting transition, crystallinity, diffusibility, film forming and emulsifying properties. As mentioned before, the wall material should be soluble in water, since almost all spray drying processes in the food industry are carried out from aqueous food formulations (Gouin, 2004). In addition, a wall material should present good properties of emulsification, film forming and drying and the wall concentrated solution should have low viscosity. Although many materials with the referred properties exist, the number approved for food uses is limited (Ghasallaoui *et al.*, 2007). Often, the selection of the wall material involves trial-and-error procedures (Pérez-Alonso *et al.*, 2003).

Microencapsulation of food ingredients can be achieved with biopolymers of different sources, such as natural gums (Gum Arabic, alginates, carrageenans), proteins (milk, whey protein, gelatin, soy protein), carbohydrates (maltodextrin, starch, corn syrups), waxes and their blends.

Gum Arabic

Gums are used in microencapsulation due to their film forming and emulsion stabilization properties. Among all gums, gum Arabic, alternatively known as acacia gum, stands out due to its excellent emulsification properties, film-forming capacities and low viscosity in aqueous solution, reasons why it is considered one of the most important encapsulating agents being widely used in microencapsulation techniques (Gharsalloui *et al.*, 2007; Silva *et al.* 2013). Additionally it is non-toxic, odourless, with a smooth taste and presents a good solubility in cold water. Because of its properties, gum Arabic has been usually used to encapsulate lipids (Kenyon, 1995; cited in Gharsalloui *et al.*, 2007).

However, the high cost of the product, low availability and quality variations have limited the use of gum Arabic for encapsulation purposes (Azeredo, 2005; Gharsalloui *et al.*, 2007).

Maltodextrin

Carbohydrates are usually used in microencapsulation of food ingredients due to their good solubility, low viscosity at high solid contents and gelling properties, which make them able to stabilize emulsions towards flocculation and coalescence (Dalgleish, 2006).

Maltodextrin shows a low viscosity at high concentrations and provide good oxidative stability but exhibit poor emulsifying capacity, poor emulsion stability and low oil retention (Azeredo, 2005; Ghasallaoui *et al.*, 2007). However, these limitations can be overcome by varying the maltodextrin concentration (Yoshii *et al.*, 2001).

1.5. Characterization of *alheiras*

1.5.1. Composition and product processing

Alheiras are traditional smoked naturally fermented meat sausages with unique characteristics, produced in the North of Portugal. Although their composition and production processes vary considerably, there are common elements in the process relevant from the food safety point of view.

The meats, provided from different sources, are boiled in lightly salted and spiced water; sliced bread is soaked in the broth formed during the previous stage; the meats cut into small pieces are mixed with olive oil, spices and the bread/broth mixture; there is no addition of starter cultures; when everything is mixed and the spices and salt contents adjusted, the mixture is stuffed into cattle intestinal or cellulose based casings; smoked for 2-8 days; and finally packaged in a controlled atmosphere or in vacuum. The shelf-life of *alheira* is 1 month, storage at 4°C in air, or longer when packed in controlled atmosphere or in vacuum. Before consumption, *alheiras* are cooked, either by frying, grilling or boiling (Ferreira *et al.*, 2006) (AESBUC, n.d.).

1.5.2. Microbiological safety and chemical characterization

The microbiological safety of *alheira* depends on a high number of parameters and characteristics intrinsic or extrinsic to the product, which influence the growth and activity of the microorganisms, including pathogens. Among others, these are the water activity, pH, amount of salt and spices, time and temperature used for the thermal processes mentioned before and the packaging conditions. Each of these factors can act as a barrier to the development of the microorganisms, limiting, retarding or avoiding the microbial growth. Commonly, the joint action of several factors is a requirement to obtain safe products (AESBUC, n.d.).

Thus, consumers generally consider *alheira* as a safe food from the microbiological point of view. However post-process contamination can occur during the addition of the bread and spices as well as in subsequent handling during filling and later manipulations and storage. Additionally, there is a chance of some microorganisms to grow in the final product if the previous mentioned parameters of the product are not enough to avoid this. Safety of traditional fermented sausages is a matter of concern for the producers and for those responsible for public health, and has been a topic of study of several research groups (Ferreira *et al.*, 2007). Talon *et al.* (2008) reported a study of the safety improvement of traditional dry fermented sausages using autochthonous starter cultures. A work of Cabeza *et al.* (2009) about safety and quality of read-to-eat dry fermented sausages reported the concern about the contamination in these products due to the size reduction included in its transformation process. Moreover, gastrointestinal disease outbreaks associated with fermented meats were reported by Moore (2004).

The most common pathogenic microorganisms associated with the production of *alheiras* are *Trichinella spiralis*, *E. coli*, *Salmonella* spp., *L. monocytogenes*, *Staph. aureus*, *Clostridium botulinum*, *Campylobacter jejuni* and *Yersinia enterocolítica* (AESBUC, n.d.).

A study of Esteves (2005) about the microbiological hazards in *alheiras*, focused on the analysis of *alheiras* from four different industries, concluded that in 70% of the samples at least one pathogenic microorganism was isolated. Among the pathogenic microorganisms being searched for, the most prevalent were *Staph. aureus*, *C. perfringens* and *Salmonella* spp. present respectively in 50%, 25% and in 12.5% of the samples.

In a study reported by Ferreira *et al.* (2006), the chemical and microbiological characterization of *alheiras* was performed with particular reference to factors relating to food safety. For that, *alheira*s from 12 different producers were analysed. Regarding to the chemical analysis, the follow mean values were reported: pH of 5.11 ± 0.5; salt content of 1.3 ± 0.3%; relative humidity of 52.3 ± 4.31%. The microbiological analysis detected *Staph. aureus*, *E. coli* and *Listeria* spp. in several samples, besides the lactic acid bacteria that comprise the major microflora of *alheira*. It allows inferring that most of the *alheiras* were produced under deficient hygienic conditions leading to post-process contamination after boiling of the meats. Moreover, according to the guidelines for the microbiological quality of fermented meats, published by Gilbert et al. (2000), most of the samples tested would be considered unsatisfactory.

A later study of the same team (Ferreira *et al.*, 2007) reported a microbiological analysis of 38 lots of *alheiras* and detected *Salmonella* spp. in 2 lots and more than 60% of the lots analysed were contaminated with *L. monocytogenes*. *L. monocytogenes* is an actual concern in food safety due to its ubiquitous characteristics and pathogenicity (Miller *et al.*, 2011).

These results indicated that despite the low pH, high salt content and reduced moisture in *alheira*, the microbiological safety of this product could be not guaranteed. Additionally, the authors found that although *alheiras* are normally cooked before consumption either by frying, grilling or boiling, the monitorization of heating profiles of this product during cooking by various consumers suggests that internal temperatures may not ensure death of the pathogens originally present (Felício *et al.*, 2011).

2. Materials and Methods

For the procedures and techniques described in the next sections, the following reagents were used: Folin-Ciocalteu reagent (Scharlau, Port Adelaide, Australia), Ringer's solution and Tryptone Soya Broth (LabM, Bury, United Kingdom), Tryto-casein Soy Agar, Mannitol Salt Agar and M17 Agar (Biokar Diagnostics, Beauvais, France), PALCAM Listeria-Selective Agar and Gum Arabic (Merck KGaA, Darmstadt, Germany), Listeria PALCAM Selective Supple (VWR Chemicals, Radnor, USA), 10DE maltodextrin (Sigma) and ethanol absolute (Panreac, Barcelona, Spain).

All the other reagents were of analytical grade and include ethanol 95% (v/v), sodium carbonate, gallic acid and glycerol.

Deionized water was used during the experiments, except when ultra-pure water is mentioned. For that, the EASY pure II, LF ultrapure water system (Barnstead, Dubuque, Iowa, USA) was used.

The propolis was collected from Vila Franca, Viana do Castelo, Portugal and was gently supplied by Samuel Jacome from *Escola Superior de Tecnologia e Gestão*, *Instituto Politécnico de Viana do Castelo*.

The main equipment used in this work were a Mini Spray Dryer B-191 (BUCHI, Flawil, Switzerland), a spectrophotometer Helios (UNICAM, Leça da Palmeira, Portugal) and an incubator (SANYO, Osaka, Japan).

For the statistical analysis, SPSS for Windows, 17.0 was used. (SPSS Inc., Chicago, Illinois, USA).

2.1. Biological material: extraction of propolis

Propolis samples from the north of Portugal, stored at -20 ºC until later use, were extracted with ethanol 95% (v/v) in the proportion of 20 g of propolis to a final volume of 100 ml, at room temperature and protected from light. The solution was stirred overnight (18h) and then remained at rest in the refrigerator for a further 24h period.

The solution was filtered through Whatman filter paper to obtain an EEP. The waste remaining in the filter was collected and a new extraction was made following the same protocol. Finally the two extracts were mixed and frozen to precipitate other compounds. The supernatant EEP was used for the subsequent assays.

2.2. Spray drying

2.2.1. Preparation of EEP carrier formulations

For the propolis microencapsulation, two different carrier materials were tested, namely, gum Arabic (GA) and maltodextrin (MD). Individual solutions of the carriers were prepared. GA and MD were dissolved in deionized water to obtain a final concentration of 1.5 and 2%, respectively. The solutions were stirred and placed in a bath at 60 ºC for 5 minutes, sterilized in the autoclave at 121 °C for 15 minutes and remained at room temperature overnight.

The previously prepared EEP was dispersed in the carrier solutions with ratios equal to 1:4 and 1:6 (w/w). The dispersion was stirred and the resulting formulations were spray dried.

2.2.2. Spray drying conditions

The formulations were atomized in a lab-bench spray dryer (Mini Spray Dryer B-191). The operational conditions of the spray dryer were as follows: inlet air temperature of 120 ºC; outlet air temperature between 78 and 82 ºC; drying air flow of 500 L/h; air pressure 6 bar; aspiration of 90%; pump of 10% and nozzle diameter of 0.7 mm. These conditions were chosen according to the work of Silva *et al.* (2013) with slight modifications due to the different characteristics of the spray dryer apparatus. In order to maintain homogeneity, while feed was pumped into the spray dryer, the

suspensions were stirred using a magnetic stirring. Fig. 3 presents a picture of the spray dryer during the process.

Fig 3. Mini Spray Dryer B-191 (BUCHI) used during the experiments of this work.

At the end of each spray drying process, the powders were gathered, weighed and placed in a plastic vessel covered with aluminium foil and kept at room temperature inside a desiccator with silica gel.

2.3. Spray drying yields

The yields of the spray drying processes were determined and expressed as the weight percentage of the final product compared to the total amount of materials sprayed. The total amount of the materials sprayed was calculated based on: the dry weight of the EEP and its volume in the formulation prepared for atomization and the % of GA or MD in the carrier solution and its volume in the initial formulation. In the powder collected after the spray drying process, the moisture content of the microcapsules was neglected.

To determine the dry weight of the EEP, inside the hotte, a sample of EEP with a known volume was added to a glass petri dish previously dried and weighted. The sample was warmed in a hot plate for a few minutes and leaved in the incubator at 100 °C for 2h. After that, the sample was weighted and leaved in the incubator for 1h. The process continued until constant dry weight was obtained.

2.4. Total phenolic compounds and total phenolic loss

Total phenolic compounds (TPC) of the EEP and the spray dried formulations were determined by the Folin-Ciocalteu method (Wettasinghe & Shahidi, 1999). This method is based on the oxidability of phenols at alkaline pH, the Folin-Ciocalteu reagent working as an oxidant agent.

Gallic acid was used as a standard for calibration curve and 0.02 g of gallic acid was solubilized in 100 mL of ultra-pure water. Different volumes of the initial solution were taken and mixed with 1 mL of Folin-Ciocalteu reagent and 10 mL of ultra-pure water. The solution was slightly stirred and 2 mL of saturated Na_2CO_3 was added. The mixture was completed with ultra-pure water until a final volume of 25 mL, obtaining standards solutions with concentrations of 2, 4, 6, 8, 10, 15 and 17 ppm. These were allowed to rest for 1 h in the darkness. The absorbance was then read using a spectrophotometer at 760 nm.

For the EEP, a sample of 15 µL was taken and the protocol was followed as described before. Triplicates were made.

From the resultant powder of the spray drying, 1 mg was mixed with 1 mL of ethanol 95% (v/v). The subsequent procedure was followed, using samples of 30, 50, 200 and 500 µL.

For determination of the phenolic loss, TPC of the EEP and of the SDPE was expressed as Gallic acid equivalents (mg GAE/100 g of sample).

To obtain a value of GAE in the SDPE per 100 g of raw propolis, the amount of dry propolis extract in the microcapsules was estimated based on: the dry weight of the EEP and the percentage of EEP in the initial formulations atomized. It was considered that the content of the microcapsules obtained was merely propolis and the carrier agent and thus the moisture content was neglected..

The phenolic loss (%) was obtained by the following ratio:

$$\frac{(\mathrm{TPC_{EEP}} - \mathrm{TPC_{SDPE}})}{(\mathrm{TPC_{EEP}})} \times 100$$

The mean value and standard deviation were calculated for each sample.

2.5. Antibacterial activity evaluation on plates

2.5.1. Origin of bacteria

Four different bacteria species were used in this study, two Gram-positive bacteria *Staph. aureus* ESBCC 81 (Culture Collection of *Escola Superior de Biotecnologia*) and *L. innocua* PHLS 2030c (Public Health Laboratory Service, Colindale, London) and two Gram-negative bacteria *E. Coli* ATCC 25922 (American Type Culture Collection) and *Salmonella* Typhimurium ESBCC 01 (Culture Collection of *Escola Superior de Biotecnologia*).

2.5.2. Growth and storage conditions

Stock cultures were grown on Trypto-casein Soy Agar supplemented with 0.6% (w/v) of Yeast Extract (TSA-YE) and Tryptone Soya broth with Yeast Extract (0.6% w/v) (TSB-YE), incubated at 37 ºC for 24h. The cultures were preserved at -20 ºC in TSB containing 30% (v/v) of glycerol.

2.5.3. Antibacterial activity evaluation

To evaluate the antibacterial activity of the microcapsules produced, the experiments were performed according to the well agar diffusion method, following two different techniques. Four bacterial strains were tested, namely, *E. coli*, *Staph. aureus*, *S.* Typhimurium and *L. innocua*. The inoculum was prepared from an overnight culture at 37 ºC on TSB-YE, by suspension of isolated colonies into sterile Ringer's solution in order to obtain turbidity equivalent to 0.5 McFarland standards.

In the first technique, 1 mL of the bacterial suspensions was pipetted into separate sterile Petri dishes to which 20 mL of molten TSB-YE with 1% agar (45 °C) were added and gently mixed. After cooling and drying, the SDPE was added following the same procedure described in the first technique. In both techniques, a control plate was made without adding the powder.

In the second technique, the bacterial suspensions described were spread onto TSA-YE plates surface with a sterile cotton swab. The swab was dipped into the broth culture and gently squeezed against the tube inside to remove the excess fluid. Then the

agar plate was streaked evenly in three directions to form a lawn. A small portion of the SDPE resulting from the different conditions was then placed on the plate.

The plates were incubated overnight at 37 ºC and the diameter (mm) of the resulting zone of inhibition was measured.

2.6. Incorporation of the inoculum and microcapsules in *alheiras*

2.6.1. Inoculum preparation

Inocula of *L. innocua* and *Staph. aureus* were prepared. Strains from the stock cultures were plated on TSA-YE and incubated at 37 ºC for 24 h. After that, one pure colony of each strain was transferred from TSA-YE to 9 mL of TSB-YE, separately, and incubated in the same conditions. This culture was sub-cultured twice (1% v/v inoculum; 24 h at 37 ºC) in 10 mL TSB.

The inocula prepared as described above were diluted in 9 mL of Ringer's solution to obtain a cell density of 5×10^8 CFU/mL and 3 mL of it were added to *alheira* according to the procedure described below.

2.6.2. Preparation of the batches

Four different experiments were carried out to optimize the proportion of microcapsules added to *alheira*. Samples from the same producer and with the same composition were collected from retail establishments.

In each experiment, two different batches were prepared, making a total of 8 batches. In all the experiments, the first batch was the control, in which only the inocula prepared as described before were added to obtain a final cell number in *alheira* of approximately 5×10^5 CFU/g of each. In the second batch, additionally to the bacteria, the microcapsules of EEP with GA (1:4) were added in the proportion of 1%, 2%, 5% and 10 % (w/w), according to the experiment. In the experiments 1 and 2 only inoculum of *L. innocua* was added and in the experiments 3 and 4, additionally to *L. innocua*, inoculum of *Staph. aureus* was added (Table 1).

The mixtures were prepared in sterile blender bags and were well homogenized. The conditions of each experiment and batch are described on Table 1. On Fig. 4, the control and propolis batches of the 4th experiment are shown.

Fig 4. Control and propolis batches of the 4t^{h} experiment prepared inside a sterile blender bag.

Table 1. Conditions of the four experiments realized.

Experiment	**Batch**	**% Microcapsules (w/w)**	**Inoculum (5×10^5 CFU/g)**
1st	Control	-	*L. innocua*
	Propolis	1	
2nd	Control	-	*L. innocua*
	Propolis	10	
3rd	Control	-	*L. innocua* + *Staph. aureus*
	Propolis	2	
4th	Control	-	*L. innocua* + *Staph. aureus*
	Propolis	5	

2.6.3. Storage conditions

In all the experiments, each batch was stored at 4 ºC during 60 days, which corresponds to the shelf-life of *alheira*. At days 0, 1, 3, 6, 10, 14, 21, 30, 45 and 60 of storage, samples were analysed for growth of the inoculated strains. Exceptionally, in the 1st experiment, on 6th day no sample was analysed and the 3rd experiment finished on 30th day once the samples finished.

2.7. Microbiological analysis

At each sampling point, samples of *alheira* were diluted in Ringer's solution in the proportion 1:10 (w/v) and homogenized (by vortexing). Serial decimal dilutions of the first one were made in 9 mL of Ringer's solution. Microbiological analysis was performed using two enumeration techniques: drop count and spread plate count. In the first one, 20 µL drops of the appropriate dilutions were spotted, in duplicate, on selective agar plates, as described by Miles & Misra (1938). In the second one aliquots of 100 µL were spread on selective agar plates.

PALCAM plates and Mannitol Salt Agar (MSA) plates were used for the enumeration of *L. innocua* and *Staph. aureus*, respectively. Plates were incubated at 37 ºC for 48 h before enumeration. The mean values and standard deviation were calculated from the data obtained from the enumeration of the bacteria. Fig. 5 shows the typical appearance of the colonies of *L. innocua* and *Staph. aureus* in its respective selective media.

Fig 5. Colonies of *Listeria* spp. on a Palcam plate (left) and colonies of *Staph. aureus* on a MSA plate (right) (from Heipha Diagnostika, available in: www.heipha.com, accessed on 17/06/2015; and Kerekesné N., available in http://slideplayer.hu/slide/2193744/, accessed on 17/06/2015.)

2.8. Sensorial analysis

Microcapsules of EPP and GA (1:4) were prepared following the same protocol as described before with the proper precautions for their use in a sensorial test. The extraction of propolis was made with ethanol absolute food GRAS.

The powder was added to *alheira* in the proportion of 5% (w/w) and well mixed. Small portions of *alheira*, the control and the one with SDPE, were cooked in the oven for 15 minutes at 200 °C.

For the sensorial analysis, a triangular test was made for a panel of 8 members not trained, aged between 22 and 76 years, selected among students and Professors of the school. It consisted in present to each person three samples of cooked *alheira*, two of

which were equal; with the purpose of identify the sample that was different (Larmond, 1977).

Since the probability of hit randomly is only 33.3%, it can be assured that the test has a good sensibility.

On Fig. 6 the survey of the triangular test is presented. The interpretation of the answers is based on binominal tables with p=1/3 (Roessler *et al*., 1978), in which the number of correct answers is added and depending of the number of persons that participate on the test, an interpretation is carried out. According to the number of participants in the test, there is a minimum number of correct answers to establish a significant difference. (Larmond, 1977; Roessler *et al*., 1978).

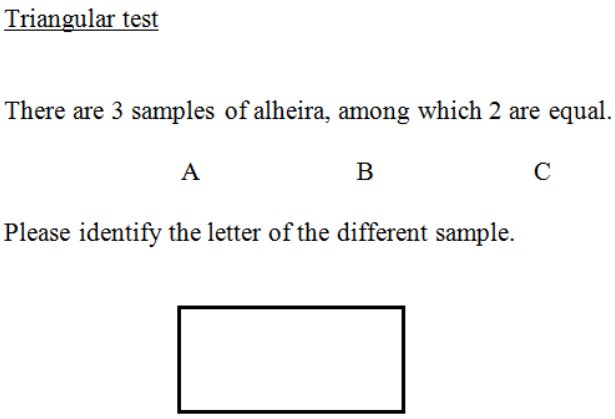

Fig 6. Survey of the triangular test, used for the sensorial analysis in this work.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was carried out to determinate significant differences within and between groups. Tukey's test was applied to compare the mean values. Statistical significance was set at $P < 0.01$. These analyses were performed using SPSS for Windows, 17.0.

3. Results and Discussion

In the next sections, the results obtained for the SDPE with GA and MD as carriers are presented and discussed, concerning to the production yield, total phenolic loss during atomization and antibacterial activity evaluation on plates. The results of the antibacterial activity of the SDPE incorporated in alheiras in different proportions and the sensorial analysis are also discussed.

3.1. Spray drying yields

The powder obtained with both carriers was fine and pale yellow-coloured (Fig. 7), as reported by Silva *et al.* (2013).

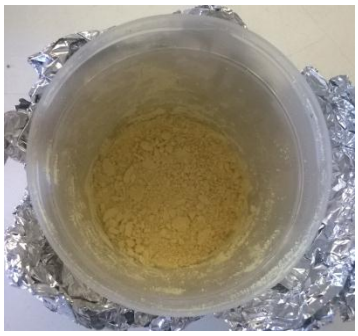

Fig 7. Sample of SDPE.

The yields of the spray drying process with the different formulations were relatively low, between 38 and 49% as presented in Table 2. Besides the formulations, the conditions of the spray drying were maintained in all the experiments. It is important to note that these values are overestimated since the moisture content of the powder was not determined and it was neglected. However, Silva *et al.* (2013) reported moisture values in EEP spray dried with GA and OSA starch as carrier between 4.9 and 12.6 %, reason why this parameter should have been determined to obtain the real value.

Table 2. Production yields of spray dried formulations.

Formulations	Yield (%)
EEP:GA 1:4	42
EEP:GA 1:6	42
EEP:MD 1:4	38
EEP:MD 1:6	49

The results are in accordance with the bibliography. Bruschi *et al.* (2003) produced propolis microparticles through spray drying using gelatin as a carrier agent, and they achieved production yields between 28 and 51%. Low yields are frequent in the spray drying method (Endo *et al.*, 2012), especially when bench equipment is used.

The formulation of EEP:MD 1:6 presented the highest yield value, while the EEP:MD 1:4 formulation showed the lowest value. This was expected since the EEP is a resinous substance and a higher amount of the carrier agent facilitates the spray drying and collection of the powder. However, for the formulations with GA, there was no difference between them. Again, since the results did not take into account the moisture content of the resultant powder, the differences observed can be due to this fact.

The low yields obtained can be attributed to the small amount of material processed but the main problem was due to the significant portion of the powder remaining adhered to the spray dryer surface inside the chamber and cyclone, and not in the collection vessel, which resulted in a very low efficiency and production. These findings can be seen in the Fig. 8.

Fig 8. Spray dryer cyclone with SDPE on the walls and not on the collection vessel.

The same phenomenon was described by Silva *et al.* (2011) in a study of spray dried propolis without the presence of a carrier agent. This was due to the resinous properties of propolis as mentioned before. It would be expectable that with a carrier agent as GA or MD this problem would be overcome but it was not.

Maury *et al.* (2005) described the effects of process variables on the powder yield of spray drying trehalose on the same model of spray dryer used in this work. It was shown that the powder yield increased with higher process temperatures, owing to improved droplet drying and reduced droplet/particle deposition on the walls of the

drying chamber. Also, increasing liquid feed flow rate or decreasing atomizing air flow rate too extensively were both detrimental to powder yield. The drying air flow rate should be as high as possible to give a high powder yield.

Finding the optimal conditions for each product to be spray dried is a trial-and error procedure that could take a long time. The results obtained may be the starting point for futures studies. Alternatives to be tested include spray drying of propolis with different carrier solutions, different ratios of propolis and the carrier agent and different spray drying conditions. An higher concentration of GA or MD in the carrier solutions was not considered since previously tests done by the research group showed that it could compromise the functioning of the spray dryer apparatus.

Additionally, in the spray drying process, when the volume of the formulations to spray dry was increased, the time of the process was increased in a non-proportional basis. With the process, the inside of the apparatus crossed by the liquid was becoming sticky and clogged, reason why it was more difficult to finish the process.

The low efficiency and production of the spray drying process was a limiting factor for the subsequent experiments.

3.2. Total phenolic loss

The phenolic compounds in the EEP and in the propolis powder obtained from the different formulations were compared in order to understand if the drying/heating occurring during spray drying were responsible for degradation of these compounds. The percentages of total phenolic loss obtained are presented in Table 3.

Table 3. Total phenolic loss (%) of spray dried formulations.

Formulations	Total phenolic loss (%)
EEP:GA 1:4	1.31 ± 0.17
EEP:GA 1:6	4.09 ± 0.16
EEP:MD 1:4	7.58 ± 0.01
EEP:MD 1:6	7.00 ± 0.16

According to the results obtained, the spray drying conditions in this work resulted in a limited loss of phenolic compounds. Comparing the two carriers tested, GA was a better protectant of the bioactive compounds during the process. It is possible that

GA, being a charged molecule, could have interacted with the polar phenolic compounds providing a themoprotective effect during the exposure to high temperatures (Silva *et al.*, 2013).

The formulation with an higher value of phenolic loss was EEP:MD 1:4.

The results obtained are in accordance with those reported by Silva *et al.* (2013). The phenolic loss of spray dried EEP with GA 1:4 and 1:6 were 3.4 and 3.0 % and with OSA starch 1:4 and 1:6 were 9.0 and 10.5 %, respectively. Additionally, González *et al.* (2009) studied the thermal stability of propolis from Tucumán (Argentina) and reported that components in propolis were stable between room temperature and 120°C. The differences found may be related to the different sources of propolis used in each study. Furthermore, the operational conditions of the spray dryer, besides the inlet air temperature, may also influence the final result.

However, different results were obtained by Marquele *et al.* (2005). The TPC recovery after spray drying of the propolis extract ranged from 45.1 to 54.9 %, only.

The determination of the TPC loss during the spray drying process was made with the aim of compare the different spray dried formulations and predict the antibacterial activity of the powder. Some studies suggest that the antimicrobial activity of propolis is associated to the phenolic compounds (Alencar *et al.*, 2007), however Cabral *et al.* (2009) reported that the activity is due to the synergic effect of various substances.

3.3. Antibacterial activity evaluation on plates

The results of the antimicrobial activity on plates containing SDPE are shown in Tables 4 and 5.

The first table concerns the first technique mentioned, in which the bacterial suspension is mixed with the molten TSB-YE 1% agar in the petri dish and the powder is adding after cooling. The second table refers to the second technique, in which a lawn of the broth culture was formed on the surface of the agar plate, before adding the powder.

Table 4. Diameter of the zone of inhibition of SDPE with different formulations tested against bacteria (*L. innocua*, *Staph. aureus*, *E. coli* and *S.* Typhimurium). Technique in which the bacterial suspension was mixed with the molten TSB-YE 1% agar in the petri dish and the powder adding after cooling.

	Diameter of the zone of inhibition (mm)			
	L. innocua	*Staph. aureus*	*E. coli*	*S.* Typhimurium
EEP:GA 1:4	5	8	10	5
EEP:GA 1:6	5	10	0	7
EEP:MD 1:4	0	10	7	0
EEP:MD 1:6	5	8	9	0

Table 5. Diameter of the zone of inhibition of SDPE with different formulations tested against bacteria (*L. innocua*, *Staph. aureus*, *E. coli* and *S.* Typhimurium). Technique in which a lawn of the broth culture was formed on the surface of the agar plate, before adding the powder.

	Diameter of the zone of inhibition (mm)			
	L. innocua	*Staph. aureus*	*E. coli*	*S.* Typhimurium
EEP:GA 1:4	10	18	10	13
EEP:GA 1:6	9	16	9	9
EEP:MD 1:4	7	18	5	10
EEP:MD 1:6	5	15	10	14

Independently of the type and ratio of the carriers, in general the SDPE presented antibacterial activity against all the tested bacteria. Negative controls were made, adding only GA and MD, and any inhibition was observed. These results qualitatively demonstrate that the SDPE has antimicrobial activity. A study of Alves *et al.* (2013) about the antimicrobial activity of propolis nanoparticles against some common meat contamination bacteria reported that the nanoparticles produced showed antimicrobial activity against *E. coli*, *Staph. aureus*, *L. monocytogenes* and *Salmonella* Thompson. Bruschi *et al.* (2003) reported that microencapsulation of propolis by spray drying preserved its activity against *Staph. aureus*.

Comparing the two techniques, in the first one it was observed that in some cases no inhibition zone was formed but in the second technique, for the same formulations and microorganisms, an inhibition zone was formed. Withal, in general, the diameter of the inhibition zone presented higher values in the second technique, which was expected

because the powder was contacting directly with the bacteria, which does not occur in the first technique. With the purpose of compare the inhibition zone between the different formulations and microorganisms, the discussion is focused on the second technique only, which presented more feasible results.

Comparing the different formulations, the results showed a higher antimicrobial activity for the formulation with propolis and the carrier with the ratio 1:4, as expected, except for the EEP:MD formulations in *E. coli* and *S.* Typhimurium. The reason is because in the same amount of powder, a higher amount of propolis is presented. Between the EEP:GA and EEP:MD 1:4, the first one showed higher inhibition zones for all the microorganisms, except *Staph. aureus*, for which a similar value was observed.

For the same formulation, there is not a consistent difference between the inhibition zone of the Gram-positive and the Gram-negative bacteria. Although the antimicrobial activity of the spray dried propolis is not extensively reported, EEP antimicrobial action has been widely reported. A study of Valença *et al.* (2011) reported the *in vitro* screening for the antimicrobial potential of a Portuguese EEP against a panel of Gram-positive and Gram-negative bacteria. The results showed that Portuguese propolis exhibits an efficient antimicrobial action against bacteria and in general, Gram-positive bacteria are more sensitive than Gram-negative bacteria, as has been widely documented for propolis samples from different origins. A study of Mirzoeva *et al.* (1997) reported the antimicrobial action of EEP and concluded that its bactericidal effect was effective against Gram-positive bacteria and only some Gram-negative bacteria. Silici & Kutluca (2005) reported that EEP samples showed high antibacterial activity against Gram-positive cocci but had a weak activity against Gram-negative bacteria. However, recently different results were obtained by Lopez *et al.* (2015), since in general Gram-negative bacteria showed to be more sensitive to red propolis extract than the Gram-positive ones, although the results varied depending on the bacteria and the propolis origin.

Despite the differences in the antibacterial activity of SPDE between the bacteria tested were not evident, the results reveal that *Staph. aureus* is the most sensitive bacteria. In the work, previously mentioned, of Alves *et al.* (2013) similar results were observed.

The unclear results of the antibacterial activity obtained can be explained according to the lack of rigor of the technique used, since the propolis powder was not weighted before being added to the plates. The main purpose of the experiment was to confirm that the spray dried propolis was able to maintain the antimicrobial activity and this was verified.

For a more rigorous and quantitative analysis, another approach should be done in future studies. The spray dried propolis can be diluted in ethanol and the antimicrobial analysis can be done according to the Miles & Misra method, above referred.

3.4. Antibacterial activity evaluation in *alheira*s

The antibacterial activity evaluation of propolis powder in *alheiras* was the main purpose of this work. Among all the formulations and after analysis of the results obtained for the production yield, TPC loss and antibacterial evaluation on plates for each formulation, EEP:GA 1:4 was selected for the following studies. The main criterion taken into account was the antibacterial activity on plates.

The percentage of powder to add to *alheira* revealed as a challenge since there are a few reported works about antibacterial activity evaluation of spray dried propolis and its addition to a food matrix like *alheira* is a new field of work providing a starting point for future studies. Therefore, different experiments were done in order to optimize the amount of powder to be added to *alheira.*

In the first experiment, the SDPE was added to *alheira* in the proportion of 1% (w/w). *L. innocua* was the only bacterium evaluated in this experiment, elected as the non-pathogenic specie to be used as a surrogate for *L. monocytogenes.*

Figure 9 shows the results of enumeration of *L. innocua* (log (n/n_0)), means and standard deviation for each sampling point, comparing the control (Lc) in which only the bacterium was added to the *alheira* with SDPE (L + P).

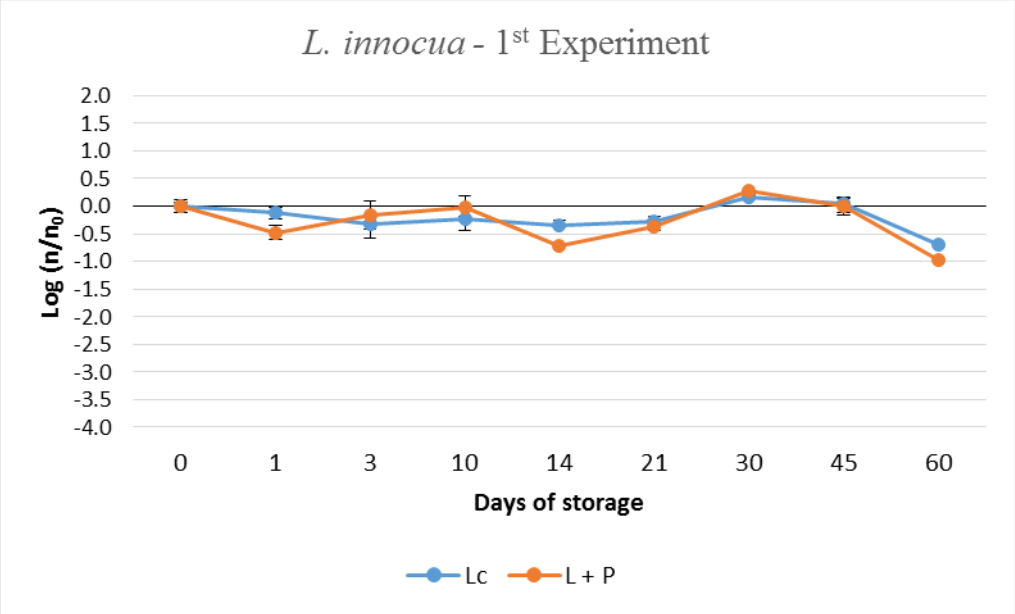

Fig 9. Enumeration of *L. innocua* in samples of *alheira* in the control batch (Lc) and in the propolis batch 1% (w/w) (L + P).

The analysis of the Fig**.** 9 shows that at each sampling point no significant differences ($P > 0.01$) were detected between Lc and L + P. Despite suffering slight variations, the levels of *L. innocua* in *alheira* remained nearly constant until the 60th day of storage in both batches. *Listeria* is able to grow in adverse conditions such as refrigeration temperatures, exhibiting a range of growth between 2.5 and 46 °C (AESBUC, n. d.), and may even survive in frozen conditions (Miller *et al.*, 2011), a pH range between 4.4 and 9 and above 13% (w/v) NaCl (Shabala *et al.*, 2008) which increases the concern about its contamination in food.

In this percentage, the SDPE added to *alheira* did not reduce its microbial load.

In the second experiment, SDPE was added to *alheira* in the proportion of 10% (w/w). After one day of storage, the counts of *L. innocua* in the propolis batch were lower than 500 CFU/g, which is the lower limit of the enumeration technique used, while in the control batch *L. innocua* counts remained constant, at the inoculated level. In the subsequent sampling points, the levels of *L. innocua* remained lower than 500 CFU/g for the propolis batch. This result suggests that the high concentration of SDPE in *alheira* was responsible for the rapid decrease of *L. innocua* in the food matrix.

Two more experiments were made with intermediate values of concentration of the powder in *alheira*, 2 and 5 % (w/w), corresponding to the third and fourth experiments, respectively. In these last experiments, beyond the study of the

antibacterial effect of SDPE against *L. innocua*, it was also tested against *Staph. aureus*. According to the evaluation on plates, *Staph. aureus* was the most sensitive bacterium to the SDPE, and therefore its study was considered.

Regarding to the 3rd experiment, the means standardized counts of *L. innocua* (log (n/n_0)) in the control (Lc) and propolis (L + P) batches and standard deviations, for a period of storage of 30 days are presented in Fig. 10.

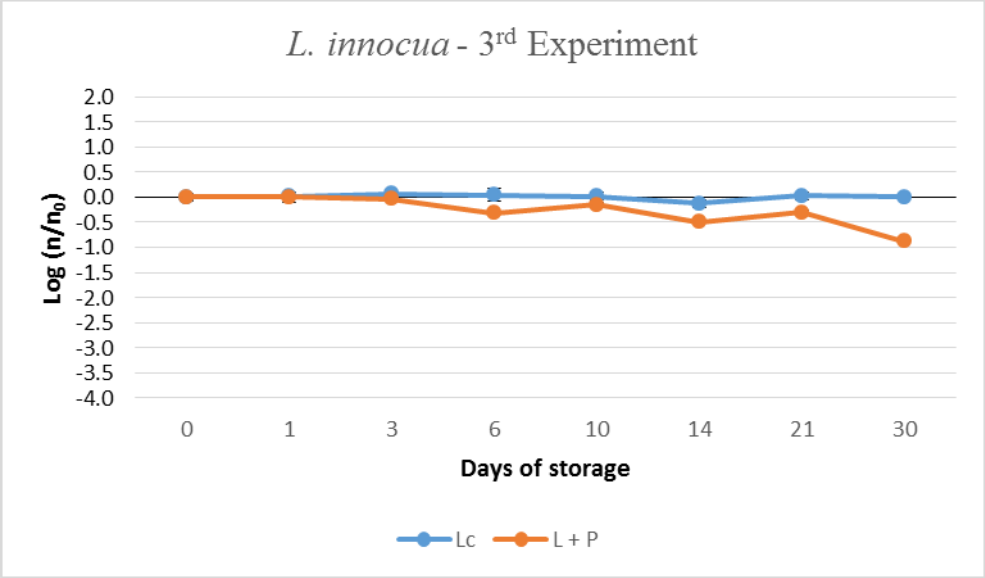

Fig 10. Enumeration of *L. innocua* in samples of *alheira* in the control batch (Lc) and in the propolis batch 2% (w/w) (L + P).

Until the 3rd day of storage, no difference in the counts of *L. innocua* was detected in both batches. From 6th day, there was a decreasing on the counts in the L + P, when compared with the Lc. However the differences observed were not significant ($P > 0.01$) and the results were not consistent between different sampling points. Only at the 30th day of storage, a significant decrease in the counts of *L. innocua* ($P < 0.01$) was observed in L + P, when compared with the previous sampling point. Nonetheless, the difference between L + P and Lc was not significant ($P > 0.01$).

A possible explanation for the decrease observed, even small, is that the release of the propolis in a spray dried form may increase after a few days of storage since, as it known, the microencapsulation provides a controlled release of the content of the microcapsule. However, this couldn't be confirmed because after the 30th day of storage no more samples were taken, once there was no more sample available.

The rate of release of the propolis spray dried is unknown and depends on countless factors. As reported by Yoshii *et al.* (2001) the flavour released from spray

dried maltodextrin/Arabic gum was markedly dependent on storage relative humidity and also on the proportion of the carrier used. It would be interesting to study these and other factors that could influence the release rate of propolis spray dried.

The standardized enumeration of *Staph. aureus* in the same experiment is presented in Fig. 11.

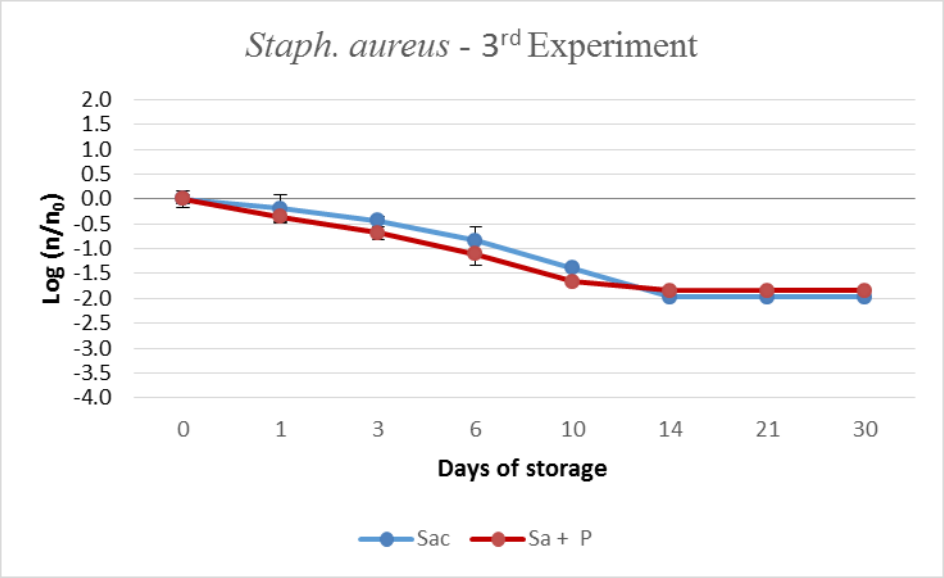

Fig 11. Enumeration of *Staph. aureus* in samples of *alheira* in the control batch (Sac) and in the propolis batch 2% (w/w) (Sa + P).

Results show that until the 14th day of storage there was a drastic decrease in the counts of *Staph. aureus* in both batches, of 2 log cycles, and the differences between those were not significant ($P > 0.01$). From the 14th day, the counts remained constant and <500 CFU/g, since it is the detection limit of the enumeration technique. The results suggest that the SDPE added to *alheira* was not responsible for an enhanced decrease of *Staph. aureus* in the batch containing propolis.

Unlike *L. innocua*, *Staph. aureus* do not grow at low temperatures, below 7 °C (Baeza *et al.*, 2009). The decreased observed may be due to several parameters that could affect the bacteria survival related with the physicochemical properties of the sample or the interaction between *L. innocua* and *Staph. aureus* since the second one competes poorly with other bacteria (Environmental Science and Research Ltd, 2001).

The results of the 4th and last experiment are presented in Figs. 12 and 13. In the Fig. 12, the standardized counts of *L. innocua* (log (n/n_0)) in the control (Lc) and propolis batch (L + P) for a period of storage of 60 days are presented.

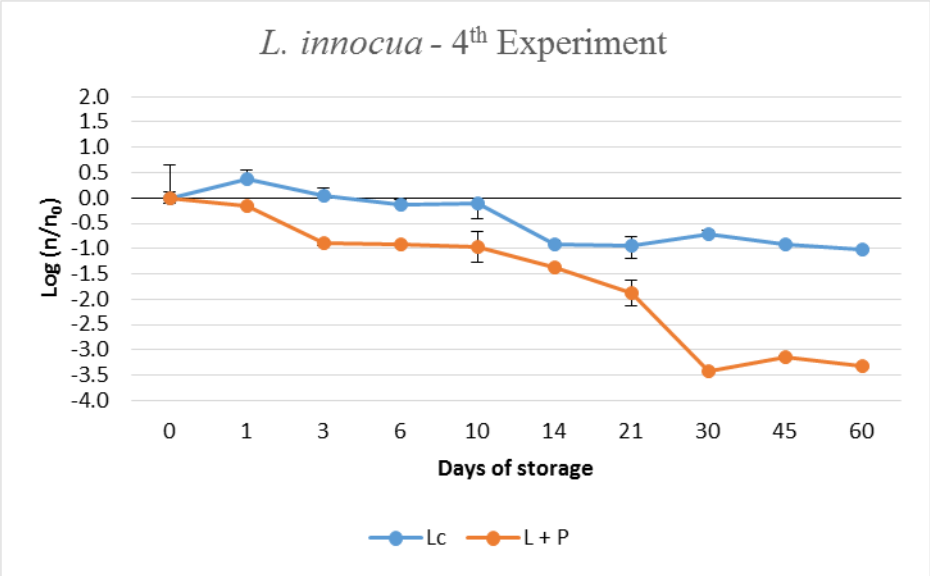

Fig 12. Enumeration of *L. innocua* in samples of *alheira* in the control batch (Lc) and in the propolis batch 5% (w/w) (L + P).

According to the results, it is possible to state that there was a significant difference ($P < 0.01$) between the sample with SDPE and the one without it in every sampling points during the storage period of 2 months, except on the 14th day. Between the 10th and 14th day of storage, the levels of *L. innocua* in the Lc decreased significantly ($P < 0.01$) compared with the initial value, moving closer to the L + P. The reasons for this decrease were not known. A possible explanation is the reduction of the pH in the sample caused by the bacteria metabolism that affects its growth, since *L. innocua* is a fermentative bacteria and produce lactic acid upon growth. A work of Conner *et al.* (1986) studied the growth of *L. monocytogenes* in cabbage juice at initial pH 5.0 to 6.1 and reported that the bacteria was capable of reducing the pH of cabbage juice to 4.14 and after that a complete inactivation occurred. However, after the decreasing of *L. innocua* in the control batch, the levels were maintained nearly constant so the subsequent results were not compromised.

Regarding to the propolis batch, the most significant ($P < 0.01$) difference occurred between the 21st and 30th days of storage. It is interesting since in the 3rd experiment, the same phenomenon was observed, even with a much lower decrease.

The difference between the two lots was very pronounced from the 21st day, with the higher difference of almost 3 log cycles in the 30th day. Thus, the efficacy of the EEP:GA 1:4 powder added in a proportion of 5% (w/w) over the control of *L. innocua* in this food matrix was demonstrated. However, the same experiment should be done in

triplicate at the same time and under the same conditions in order to confirm the results obtained.

Regarding the control of *Staph. aureus*, the results are shown in Fig. 13.

Unlike the results observed in the 3rd experiment, a decrease in the viable counts of *Staph. aureus* did not occur immediately after the beginning of storage at 4°C. Due to the differences in the results obtained in the 3rd and 4th experiments, more tests should be done in the future to understand the conditions affecting *Staph. aureus* growth.

Between the Sac and Sa + P, a significative difference ($P < 0.01$) was observed for all the sampling points, except the day 0. The efficacy of the SDPE against *Staph. aureus* was more evident after the 10th day of storage, with differences between the Sac and Sa + P of 2, 3 and 2 log cycles in the 14th, 21st and 30th days of storage, respectively. In the time period comprised between the 10th and 30th day of storage the presence of SDPE in *alheira* had a positive effect in the control of *Staph. aureus*. After the 30th day, in the following sampling points, the counts of *Staph. aureus* were under the detection limit of the technique used for enumeration.

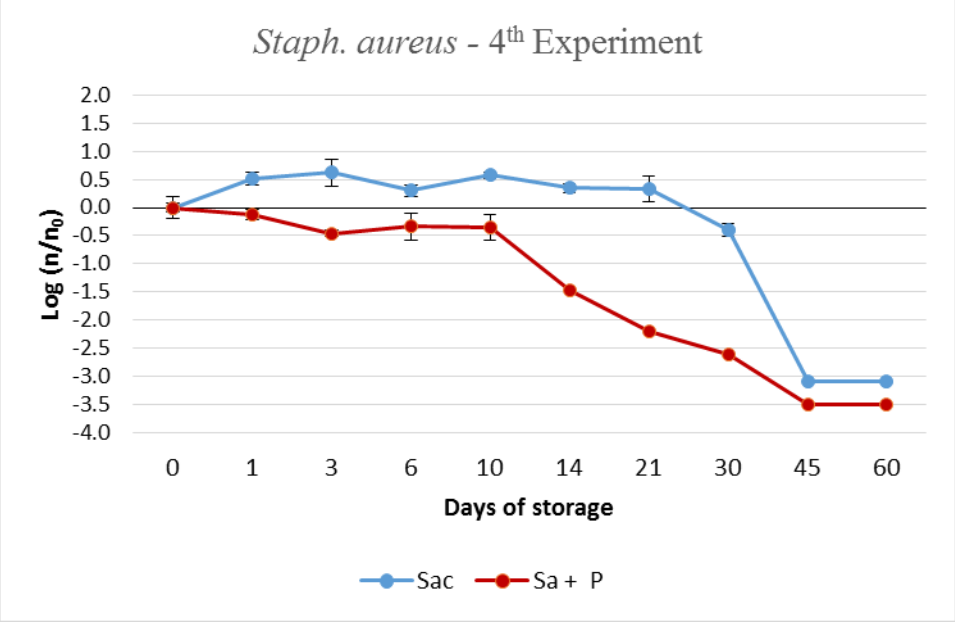

Fig 13. Enumeration of *Staph. aureus* in samples of *alheira* in the control batch (Sac) and in the propolis batch 5% (w/w) (Sa + P).

The results of the 4th experiment are very optimists since the SDPE was effective in the control of *L. innocua* and *Staph. aureus*.

3.5. Sensorial analysis

The sensorial analysis of the presented work was a preliminary test due to the small size of the panel. It was important to set the course for future experiments.

The results of the triangular test showed that 100% of the participants answered correctly, which means that all the participants identified correctly the sample that was different from the other samples. This allowed concluding that there was a significant difference between the *alheira* with and without propolis powder ($P < 0.01$).

The triangular test is a discriminant trial, aiming to establish the difference between two samples, regardless of the peculiarities of each of these. Due to the results of this test, a descriptive trial was not considered.

However, a comment about the difference of the samples was asked to the participants in the trial and the most common one was that the *alheira* with propolis powder was bitter. One of the participants considered that the *alheira* with propolis was rather saltier, which was not an expected result.

The results obtained allowed to conclude that the incorporation of EEP:GA 1:4 powder in alheira in the proportion of 5% (w/w) is not suitable for application, since the properties of this food are changed, which is undesirable and it is not the purpose. Other alternatives should be tested.

4. Conclusions

In the presented work, microencapsulation of EEP was achieved by spray drying and made it possible to obtain propolis in a powder form. The production yield of the different spray dried formulations, namely EEP:GA and EEP:MD at ratios of 1:4 and 1:6 (w/w), presented values in a range of 38-49%, the lower for EEP:MD 1:4 (v/v) and the higher for the same formulation with a ratio 1:6 (v/v). The TPC loss during the atomization process had a lower value of 1.31 ± 0.17% for the EEP:GA 1:4 and a higher value of 7.58 ± 0.01% for the EEP:MD 1:4. This demonstrated that phenolic compounds are preserved during spray drying of propolis .

The SDPE exhibited antibacterial activity against the Gram-positive bacteria *L. innocua* and *Staph. aureus* and the Gram-negative *E. coli* and *S.* Typhimurium. Among all, *Staph. aureus* was considered the most sensitive bacterium.

Considering the production yield, TPC loss and the antibacterial evaluation on plates, the spray dryer of propolis with GA at a ratio of 1:4 gathered the best results.

The antibacterial activity of the SDPE, resultant from the EEP:GA 1:4, against *L. innocua* and *Staph. aureus* in *alheira* was evaluated. Incorporation of SDPE into *alheira* in the proportions of 1 and 2 % (w/w) showed non-significant differences ($P > 0.01$) in the behaviour of the bacteria. The SDPE added to *alheira* at a ratio of 5 % (w/w) revealed to be efficient in the control of *L. innocua* and *Staph. aureus*, since survival of these bacteria during storage decreased significantly ($P < 0.01$). However, the main purpose of masking the strong and bitter flavour of propolis, while exhibiting antimicrobial activity in *alheira* food matrix, was not achieved.

Although the results obtained are very promising, since the antibacterial activity of propolis in a powder form added to *alheira* was demonstrated, further studies should be done in order to assure that the organoleptic properties of this food are not changed by the presence of propolis.

5. Considerations for future work

The development of this work set the bases for futures studies in this field. Regarding to the SDPE, it would be interesting to evaluate the physicochemical properties of the microcapsules such as the moisture content and water activity, water dispersibility, hygroscopicity, particle size measurement, density, among others. Also, the morphological characteristics of the microparticles should be considered in order to understand if the microencapsulation was well succeeded and the propolis was mostly inside the carrier. It is important to better predict the subsequent results of the experiments.

Besides loss of the phenolic compounds during drying, the stability of these during storage may be considered, since one advantage of the spray dryer process is to ensure the stability of the compounds over the time.

Additionally, other carrier agents and operational conditions of the spray dryer may be tested to obtain a higher yield and to optimize the physicochemical properties of the resultant powder.

With the aim of better understanding the effect of the SDPE added to *alheira* in the behaviour of *L. innocua* and *Staph. aureus*, the physicochemical properties of the food matrix should be analysed and monitored over the time. Moreover, the release kinetics of the SDPE and the conditions affecting it would be an added value for future developments.

Finally, the bitter flavour of propolis when incorporated in *alheira* should be masked, which was the main purpose of the presented work. Besides other carrier agents that may be considered, other percentage of the powder in *alheira*, in addition to the ones studied in this work, should be tested. Also, it would be interesting to study the combination of propolis with other natural substances that could be able to cause a synergic effect and, at the same time, mask its flavour.

Once finding the proper way of incorporate propolis powder in *alheira* contributing to its microbiological safety, it is important to evaluate the inherent toxicity of this natural product at the concentrations considered, before suggesting its use in industrial applications.

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